EVALUATION OF MICROWAVE IRRADIATION AS A METHOD FOR STERILIZING PLASTIC TISSUE CULTUREWARE AND FOR VIRAL VACCINE PRODUCTION

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CHAPTER I

LITERATURE REVIEW

Introduction to Microwave Irradiation

Microwaves are very short electromagnetic waves, covering the interval between visible light and radio-frequencies, and are classified as non-ionizing radiation. In general, radio-frequencies extend from direct current up to the infrared region. The shortest wavelengths or highest frequencies of the radio spectrum are in the microwave region. The true boundaries of microwaves vary. The United States of America Standards Institute considers the frequencies 10 and 100,000 MHz as the microwave region (3).

The measure of electromagnetic waves depend on the following set of parameters: frequency, electric field intensity or magnetic field intensity, and oscillation. Usually electromagnetic waves propagate with a given velocity and have a determined length. When the electromagnetic waves travel (propagate) through a given medium, the interrelated changes of the waves (velocity, length, and frequency) rely on the electric and magnetic properties of medium. Differences in the dielectric properties (a physical characteristic of the materials) of target materials affect the condition of propagation and absorption of microwave energy as it penetrates the surface of a subject (3).

Microwave energy is absorbed by subjected materials because of the short wavelength and consequent compact circuitry of microwaves. Since

microwave energy has a heating effect just as other forms of energy, it has been widely used on a consumer level in ovens for home cooking.

Effects of Microwave on Bacteria

The early literatures contain many references to the effects of radio-frequency (R-F) fields on bacteria (7, 8, 15, 16, 18, 26). Some indicated lethal effects on bacteria from relatively weak fields (16, 26); some indicated a complete absence of lethal effects (7, 18). The most interesting questions developed from this research have revolved around what the actual effects were, heat or irradiation.

Fabia *et al*. (15) exposed 20 ml of *E. coli* suspensions under 7.5, 10.0, and 15.0 mc/sec energy in a condenser-cooler apparatus which maintained the medium at about 19° C by running cold water through the jacket of the condenser. They found about 88% of the *E. coli* cells were destroyed after 8 hours treatment at 10 mc/sec R-F energy.

Fleming (16) treated 10 ml of *E. coli* suspensions at 11 to 350 mc/sec R-F energy. Input power was 10-W. The time of exposure was 1 minute for all treatments. The lethal effects on bacteria occurred at 60 mc/sec and about 98% of *E. coli* were destroyed.

Nyrop (26) introduced electrodes directly into *E. coli* suspensions at 10-100 Kc/sec of R-F energy. Kill was achieved in 5 to 10 seconds exposure. There was no difference in results between the temperature at 12-40 and 40-60° C.

Jacobs *et al*. (18) did many experiments on *E. coli* and *S. aureus* with R-F energy of frequencies from 12 to 66 mc/sec. In most of their experiments, no significant destruction of the bacteria was observed. The maximum temperature in the samples was about 32° C.

Brown et al. (7) investigated the effect of R-F energy at 50 c/sec, 190 kc/sec, and 26 mc/sec on E. coli. The bacterial suspensions were irradiated in a capsule electrode assembly. They found that the destruction of E. coli occurred only when temperature reached 55° C.

Olsen et al. (28) have studied unpublished data of Baker et al. They found that in Baker's experiments, microwave energy at 2,450 mc/sec inhibited the germination of macrospores of Fusarium solani var. phaseoli. Olsen (27) and Olsen et al. (28) also found that bread mold fungal spores could be eliminated by treatment with microwaves on a conveyor assembly. The bread was brought to 65° C in 2 minutes and cooled to room temperature. When spores of the same fungus were exposed to the same temperature by conventional heating, there was no reduction in spore viability. They concluded that R-F energy has a nonthermal effect on spores.

Goldblith (17) performed quantitative studies, showed that survival data obtained in the heating of E. coli suspended in PBS at 2,450 MHz microwave energy were nearly identical to the expected survival curve based on the data obtained in the conventional heating. An additional set of experiments was performed with different amounts of ice added to the E. coli suspension prior to heating in the microwave. The ice was added to the bacterial suspension to obtain a longer exposure time while maintaining a lower overall temperature. He concluded that the inactivation of E. coli, when exposed to microwave irradiation, appeared to be due to the heat. The same conclusion was drawn from similar work with B. subtilis spores.

Lechowich (20) used a different device also showed that the death of Streptococcus faecalis and Saccharomyces cerevisiae cells upon

exposure to microwave was brought about thermal rather than nonthermal effects. The microorganisms were contained in the central tube of a modified Liebig condenser placed in the approximate geometric center of the modified commercial 2,450 MHz microwave oven cavity. One of the most important parts of this experiment was that they used kerosene as a heat exchange medium. Kerosene has a low dielectric constant and does not absorb an appreciable quantity of microwave energy. The whole experiment was held at 25 to 55° C during various time periods of microwave exposure. Conventional heat experiments were also done at temperatures ranging 45 to 55° C in a water bath. Continuous application of microwaves to suspensions of 10^8 to 10^9 Streptococcus faecalis or Saccharomyces cerevisiae cells per ml appeared to produce no lethal effects other than those produced by heat.

Culkin et al. (11) claimed that from their data the heat generated during the microwave exposure was not the only reason for the nature of the lethal effects of microwaves for microorganisms in soups. In their experiments a 500 ml graduate cylinder with five short side-arms attached to the cylinder at regular intervals was used as a container. Soup and bacterial mixture was filled into cylinder and exposed to microwave irradiation. Slight differences were shown in the 3 portions (top, middle, and bottom). After microwave irradiation at 915 MHz, organisms in the bottom portion had the greatest survival levels, organisms in the top portion had the lowest survival levels. When compared to the temperature data it was obvious that the temperature in the bottom portion was higher than the temperature in the top portion. So the conclusion was made that the heat generated during the microwave exposure alone was inadequate for killing organisms.

Thermal effects (due to heat generated) of microwaves on biological systems certainly occur. Whether non-thermal effects also occur has been under debate, partly because of difficulties in designing experiments to uncover nonthermal effects in the presence of thermal effects, and partly because of lack of clear reasons in physics or chemistry for one to expect nonthermal effects. Cope (10) has suggested that superconductivity in biological systems offered reasonable basis for possible nonthermal effects of microwaves on biological materials.

Latimer et al. (19) reviewed those early experiments and found that most of the reports have shown microwaves to be effective in the killing of microorganisms. He proposed that practical applications for the irradiation effect of microwave ovens on bacteria might be used in the clinical microbiology laboratory. Different bacteria, including spore formers, in 5 ml broth cultures were subjected to microwaves at 2,450 MHz for various time periods. Wild-type isolates were included as control organisms. The results indicated that bacteria and spores were destroyed within 60 seconds and 11 minutes respectively. Wild-type isolates were killed by a 5 min exposure to microwaves. They suggested that microwave irradiation could be an appropriate method for decontamination of clinical specimens. According to their observations, microwave irradiation would not be suitable for use in the sterilization of newly prepared media because of the lack of pressurization within microwave ovens. It also was not recommended to use for sterilization of dry, wrapped materials because of possible ignition under microwave exposure. Another problem was that microwaves did not heat glass without the presence of moisture, therefore sterilization of glass pipettes seemed impractical.

Microwave energy has also been used for food sterilization and for the fixation of fetal as well as surgical and autopsy materials (5, 22, 34). Patterson and Bulard (32, 33) have demonstrated that microwaves were capable of fixing cells in tissue culture as a substitute for conventional chemical fixation methods.

Sendai Virus Infections in Mice

Sendai virus has been recognized as an infectious agent in many mouse colonies. Its usual biologic feature has been high infectivity with little overt clinical disease. Andrewes et al. (1) have found that newly isolated strains of Sendai virus rarely produced illness when inoculated into the lungs of mice, although virus replication could still be detected. After serial passage, the virus frequently produced consolidation of lung resulting severe illness and death. Similar results have been demonstrated by Robinson et al. (38).

The full potential of Sendai virus as a respiratory pathogen has been noticed recently. The major target organ for Sendai virus was the lung. However virus could be isolated readily from the saliva and some times from other organs (30). Robinson et al. (38) reported that intranasally-infected mice developed pulmonary lesions in the absence of clinical disease. These included necrosis of bronchiolar epithelium, interstitial pneumonia, and a mixed infiltrate of polymorphonuclear leukocytes and macrophages.

Parker (31) believed that Sendai virus was the leading cause of pneumonia in mice and together with the hepatitis viruses, it was the most prevalent and important of the naturally occurring virus infecions

of mice. The host range of Sendai virus includes rat, hamster, mouse, and guinea pig (36, 37, 41).

Some experiments of intranasal inoculation of non-immune mice indicated that susceptibility depended on age and amount of infections virus inoculated. Younger animals were more prone to fatal infections, but beyond 4 weeks of age there no significant age-related differences in morbidity and mortality (4, 39). Strains of mice played another important role during the infections. In colonies the infection usually persists as long as susceptible mice are present.

Transmission studies were shown that when a group of susceptible mice came in contact with infected mice they rapidly became infected. Virus was isolated from lung specimens for about 12 days and from saliva specimens for about 9 days after contact. Parker (31) suggested that the primary method of transmission was fomites contaminated by infected mice. CF and HI antibodies usually could be detected from infected mouse colonies.

Previous data hvae indicated that microwave irradiation is an acceptable method to kill bacteria and molds (16, 26, 19), which attracted us to conduct the similar experiments to confirm that microwave energy will destroy bacteria and will inactivate viruses by using 2,450 MHz commercial microwave oven in our laboratory. Since there is no suitable technique for recycling disposable plastic tissue cultureware, which is the item of greatest expense in tissue culture work, the evaluation of the decontamination of microwave irradiation of plastic ware for reuse may provide a possible method to sterilize used tissue cultureware. Also, due to the quick heating ability of microwave energy,

the effects of microwaves on antigenicity of viruses was studied as a substitute for conventional heating technique for heat killed viral vaccine production.

CHAPTER II

MATERIALS AND METHODS

Balanced Salt Solution Without Ca^{++} and Mg^{++} (BSS) 10X

BSS-10X was made by mixing 80 gm of NaCl, 4.0 gm of KCl, 3.5 gm of $NaHCO_3$, 10.0 gm of glucose, and 0.2 gm of phenol red in one liter of distilled water. The 10X solution was stored at room temperature with the addition of 5 ml of cholorform. The stock solution was diluted to 1X and autoclaved before using as a washing solution for the cells or as a diluent for viruses.

Phosphate Buffered Saline (PBS) 10X

PBS-10X stock solution was prepared as followed: 55.9 gm of Na_2HPO_4, 27 gm of KH_2PO_4, 41 gm of NaCl and then brought to a volume of 1 liter with distilled water. The stock solution was diluted 1 : 10 in distilled water and the pH was adjusted to 7.2 before use.

Trypsin-EDTA Solution

Trypsin-EDTA, the enzyme solution used to dissociate cell monolayers for passage and to disrupt tissue for primary cell culture, was made by mixing of 0.2 gm of 1 : 250 trypsin (DIFCO LAB, Detroit,

Michigan), 0.2 gm of Na_2EDTA and one liter of BSS. The solution was gently mixed at 4° C for 2-3 hours, pH was adjusted to 7.2, then filter sterilized. The solution was stored at -20° C.

Growth Medium 1X, 2X, MEM or BME

Dry powdered tissue culture media, Minimal Essential Medium (MEM) and Basal Medium Eagle (BME) (KC Biological, Kansas City, Kansas), both without $NaHCO_3$ and with L-glutamine, were the base for the growth media. Hank's base MEM with 10% serum was made by mixing 10.62 gm of MEM and 1.0 gm of $NaHCO_3$ in 900 ml of distilled water and 100 ml of new born bovine serum (KC Biological, Inc., Kansas City, Kansas). BME with 5% serum was made by mixing 10.3 gm of BME and 5.0 gm of HEPES (N-2-hydroxyethlpiperazine-N'-2-ethanesulfonic acid) in 950 ml of distilled water and 50 ml of serum. To both media 0.06 gm of sodium penicillin G and 0.1 gm of streptomycin sulfate were added, the pH were adjusted to 7.2 using 5 N NaOH, and the media were filter sterilized and frozen at -20° C until used.

The 2X growth medium contained the same components as 1X growth medium; the total volume was adjusted to 500 ml.

Nutrient Agar Overlay

First overlay: 2X 5% Hank's MEM was brought to a temperature of 46-47° C and mixed with an equal volume of melted 2% Bacto agar (at 46-47° C) (Difco Lab, Detroit, Michigan). The nutrient agar overlay was held at 46-47° C during addition to the culture. For BME, 1.4% Noble agar (Difco Lab, Detroit, Michigan) was used.

Second overlay: 1 : 300 neutral red solution (ISI Biologicals, Cary, Ill.) was added immediately before use to first overlay at 46-47° C to make the final concentration of neutral red to 1 : 12,000.

Alsever's Solution

Alsever's solution was used in collection and storage of chicken and sheep blood used in Hemagglutination and complement fixation assays. It was prepared by mixing glucose (2.05 gm), Sodium citrate (0.8 gm), NaCl (0.42 gm), Citric acid (0.02 gm) and 100 ml of distilled water. The solution was stored at 4° C. Whole blood was diluted 1 : 1 with Alsever's solution.

Red Blood Cell (RBC) Suspension

Standard solutions were made by collecting whole chicken or sheep blood in Alsever's solution, centrifuging at 1700 rpm in an International Clinical Centrifuge Model CL for 5 minutes, and washing the cells 3 times in PBS. The cells were resuspended to a concentration of 0.6% packed cells/vol. for HA assay and 1% for CF assay.

Passage of BGMK Cell Line

BGMK cells were obtained from G. Wallis, Baylor Medical Center, Texas. Cells had been passaged for 68 times, and maintained in 5% Eagle's MEM (Hank's base) in disposable flasks.

The nutrient medium was first decanted and the cells were washed with sterile BSS. Then 10 ml of trypsin solution was added and cells were incubated at 37° C for 5 minutes. When the cell sheets detached from flask surface, cells with trypsin solution were poured into small

test tubes and were separated in a clinical centrifuge at switch set 4 for 5 minutes. The pellet was resuspended in 10% MEM and distributed to 75 cm^2 tissue culture flasks. Passage ratio was 1 to 3.

Chicken Embryo Fibroblast (CEF) Cells

Ten to twelve old fertile chicken eggs were soaked in alcohol (95%) and exposed to ultraviolet light in a Bioquest Biological Cabinet (Laminar airflow sterile hood). The healthy embryos were aseptically removed from the eggs with curved forceps and placed in a sterile plastic petri plates. The embryos were decapitated and eviscerated and transferred to a sterile petri plate which contained 30 ml of sterile BSS. The washed bodies were placed in the tube of a sterile 30 ml syringe and expressed into a 125 ml trypsinizing flask by exerting pressure on the plunger. Trypsin-EDTA solution was then added to the macerated tissue. The tissue solution was stirred at room temperature for 30 minutes. The supernatant was poured into a sterile 15 ml screw-capped test tube. Cold trypsin-EDTA (50 ml) was added to the remaining large pieces of tissue and the stirring was resumed. At the same time, tubes containing the supernatants (cell suspensions) were centrifuged at switch setting 4 for 5 min in a clinical centrifuge to pellet the cells. Upon completion of centrifugation, the trypsin-EDTA supernatant was poured off and replaced by a minimal amount of sterile 5% BME. The cells were then resuspended by vortexing and filtered through sterile gauze into a sterile 1 liter flask. This procedure was repeated until desirable amount of cells obtained or no large pieces of tissue remained. The concentration of cells in the BME was determined by direct count using a haemocytometer. The cell suspension was adjusted to

1.0×10^6 cells/ml, then seeded in the appropriated tissue culture flasks. Cultures were incubated at 37° C.

Poliovirus Plaque Assay on BGMK Cells

The following procedure was a minor modification of the technique originally described by Hsiung and Melnick (21). Growth medium was removed from BGMK cell monolayers in 60 x 15 mm petri plates (COSTAR, Cat #3060, Cambridge, MA). Monolayers were washed once with sterile BSS. The diluted virus suspension was inoculated onto the monolayers in a volume of 0.1 ml in duplicate plates. The inoculum was distributed over the monolayer and the cultures were incubated at 37° C tissue culture incubators for 1.5 hours to permit virus adsorption. The cells were washed once with BSS to remove unadsorbed virus particles prior to adding the overlay.

First overlay was maintained at a temperature 46-47° C. Each culture received 4 ml of the overlay which was spread carefully over the monolayers. The plates were placed on a flat surface for 30 minutes for the agar to harden then they were inverted and incubated in the dark at 37° C tissue culture incubators. Plaques appeared 48 hours post-infection.

Isolation and Purification of Polioviruses

Polioviruses were obtained from the Student Health Center of Oklahoma State University. The specimens were isolated from the mouth of a volunter with a swab. The sample was eluted in 5 ml of sterile BSS solution, then passed through 0.45 µm disposable filter (ACRODISC, Gelman Products No. 4148). The suspension was inoculated onto BGMK cell

monolayers. Cytopathic effect (CPE) developed at 72 hours. Viruses were passaged 3 times. Ten fold serial dilutions were made from the spent growth medium and 0.1 ml of each diluted sample was inoculated to duplicate plates. By using standard plaque assay for polioviruses, three different sized plaques were picked (large, medium, and small) from infected plates. The agar on top of each plaque was removed by disposable Pasteur pipettes and suspended in BSS. The suspensions were inoculated on BGMK monolayers and replaqued. The plaquing procedure was repeated twice. Individual plaques were picked as purified poliovirus for serological identification.

Identification of Polioviruses

Poliovirus isolates were of three distinct immunological types, which can be recognized serologically by virus neutralization. The LIM BENYESH-MELNICK serum pools were used for virus identification (35). Briefly, serum pools were rehydrated in double distilled water. Each pool was diluted 10-fold. A 0.5 ml aliquot of the serum was allowed to react with 0.5 ml of unknown poliovirus for 2 hours at 37° C. A 0.5 ml aliquot of the mixture was added to BGMK monolayers in duplicate. Virus control plates received a 0.25 ml aliquot. All plates were incubated at 37° C for additional 1.5 hours, then the plaquing method was performed. Plates were checked for 7 days.

Poliovirus Stocks

After a monolayer of BGMK cells were formed, they were infected with the polioviruses. The nutrient medium was poured off, cell sheets were washed with BSS, and 1.0 X 10^{6-7} PFU of virus suspension was

added. The flasks were incubated at 37° C for 1.5 hours for the virus to attach to the cells. After the incubation, MEM medium without serum was added to the cells and reincubated at 37° C until complete CPE was formed. The virus suspension with infected cells was harvested by freezing and thawing 3 times, then stored at -20° C.

The cellular debris was removed by centrifugation at 10,000 rpm for 15 min in a Sorvall SS-34 rotor. The supernatant was clarified for 3 hours at 23,000 rpm in a Beckman SW-28 rotor. The virus pellet was resuspended in 3 ml of PBS. Virus suspension was centrifuged over a discontinuous (15, 30, 45, 60 percent w/v) sucrose gradient at 20,000 rpm for 2.5 hours in a Beckman SW-28 rotor. The gradient showed two bands at the boundary between 60% and 45% sucrose gradient which were collected separately using a bent needle and syringe. The collected virus preparation was dialysed against PBS buffer to remove the sucrose. Viral activity was monitored by a plaque assay on BGMK cells. The virus stocks were frozen at -20° C until used.

Hemagglutination Titration (HA)

Hemagglutination titers of Sendai virus were determined in plastic disposable trays. Standard procedures for HA assay were used (23). A virus sample (0.025 ml) was added to the first well and a serial two-fold dilution was made in PBS. Chicken red blood cells (0.6%) suspension in PBS was used as an indicator. The trays were incubated for 1 hour at room temperature and the viral HA titer was considered to be the inverse of the dilution of virus in the well showing 50% hemagglutination.

Hemagglutination Inhibition Assay (HI)

Mouse serum samples were tested to determine if they contained antibodies against Sendai virus. The procedure used was that described in the Manual of Clinical Immunology (23).

Sendai Virus Stock

Sendai virus, OSU-T strain, was isolated by M. R. Sanborn, Dept. of Microbiology, Oklahoma State University. The exact passage history of the strain is unknown; however, the strain was isolated from a chicken strain provided by D. W. Kingsbury at St. Jude Research Center in Memphis, Tenn. The OSU-T strain has been propagated in turkey eggs for 5 years.

OSU-T strain Sendai was propagated in 14 day-old embryonated turkey eggs. The turkey eggs were candled to determine fertility, then eggs were cleaned by 75% alcohol. A small hole was placed in the blunt end of the eggs, and a sterile 0.2 ml of stock solution (4 HA/0.2ml) of infectious allantoic fluid was injected into the allantoic sac. The eggs were incubated at 31° C for 48 hours, and refrigerated at 4° C overnight before the allantoic fluid was harvested. The crude allantoic fluid was stored at -20° C.

Allantoic fluid was clarified by low speed centrifugation at 5,000 rpm for 15 minutes in a Sorvall SS-34 rotor. The supernatant was made 0.5% with NaCl and with 6.0% PEG (polyethylene glycol, 6,000) and was incubated at 4° C overnight. The mixture was centrifuged in Sorval RC-2 at 6,000 rpm for 15 minutes. The pellet was collected and resuspended in a small amount of PBS and layered onto a discontinuous sucrose gradient (9.5 ml of each 15, 30, 45, and 60 percent w/v) in a SW-28

rotor at 20,000 rpm for 2 hours. The opaque bands were extracted with a syringe and curved 16 gauge needle. The virus was concentrated again by dialysis and resuspended in a minimal volume of PBS. The virus fraction was then layered onto a continuous sucrose gradient (24-58% w/v) formed by Beckman Linear Gradient Former and centrifuged at 20,000 rpm for 2 hours in SW-28 rotor. The opaque bands were collected as mentioned above. Virus suspensions were dialysed against PBS overnight to remove sucrose then viruses were stored at -20° C.

Plaque Assay of Sendai Virus

The OSU-T strain Sendai to be assayed was filter sterilized by passing the purified virus through a 0.45 μm filter (Gelman). The virus was aseptically diluted serially in BSS and 0.2 ml of each dilution was added to a confluent monolayer of CEF cells. The virus was allowed to attach at room temperature for 30 minutes and 5 ml of BME first overlay was added to each plate. Plates were incubated at 31° C for 48 hours then 5 ml of second overlay per plate was added. Plaques were observed by 60 hours post-infection.

Complement Fixation Test (CF)

The complement fixation test was used to detect the CF antigen of polioviruses and anti Sendai virus antibodies. The procedure used was described in the Manual of Clinical Immunology (23).

Preparation of Bacteriophage T_4

E. coli B was grown in nutrient broth with 0.5% NaCl. A 10 ml volume of 10^6 PFU of T_4 phage suspension was added to the exponentially growing cells. T_4 particles were harvested after 18 hours propagation and concentrated as described for polio viruses.

Bacteriophage T_4 was detected by a plaque assay. A 0.5 ml aliquot of the mixture (equal volume of E. coli B cells and T_4 particles) was added to 5 ml of soft agar (0.5% NaCl, 0.75% Bacto agar in Nutriment Broth) held at 45° C. Soft agar containing the cell-phage mixture was poured onto a 0.5% NaCl nutrient agar plate in duplicate. Plates were incubated at 37° C for 18 hours then plaques were scored.

Microwave Irradiation for Sterilizing Disposable Tissue Cultureware

A locally purchased Kenmore (model 99601) microwave oven operating at 2,450 MHz with power selector on HI was used throughout the experiments. Exposure times were controlled by the built-in digital timer.

Tissue cultureware was purchased from several sources including several brands and vessel sizes: Corning 5 cm^2 tissue culture flasks, Corning Glass Works, Corning, New York; Costar Tissue Culture Cluster 24 and 60 X 15 mm dishes, Costar, Cambridge, Mass; Lux 25 cm^2 tissue culture flasks, Lux Scientific Corporation, Newbury Park, California; and Falcon 850 cm^2 Tissue Roller Bottles, Falcon Labware, Oxnard, California.

Sterile vessels were contaminated with 0.2-5.0 ml of a stationary phase culture of the following organisms: Pseudomonas fluorescens,

Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Sarcina lutea, Corynebacterium equi, Bacillus alvei, Bacillus globigii and Streptococcus faecium. Contaminated vessels were subjected to microwave irradiation for the following times: 0, 15, 30, 60, 120, 180, 240, 300, 600 seconds. After irradiation the contaminated surface of the vessels were overlayed with nutrient agar. The vessels were then incubated at 37° C for 48 hours. Each container was then scored for the presence or absence of bacterial growth.

Non-sterile, used, vessels were washed with Alconox detergent and rinsed with deionized water from a Barnstead NANO-pure water system. After overnight drying the vessels were subjected to microwave oven as described above. Each vessel then received an aliquot of tissue culture medium and was incubated at 37° C for 48 hours. Vessels were then scored for contamination by observing the medium for a pH shift and also microscopically with Gram stains.

Further experiments were conducted to determine if used tissue culture flasks could be washed, sterilized with microwave irradiation, and reused. Used flasks were washed with detergent to remove attached cells and rinsed with deionized water thoroughly. After flasks were completely dried they were submitted to microwave irradiation and were seeded with 1×10^6 cells/ml the following cell types: primary chicken embryo, primary turkey embryo, Hep-2, VERO, BGMK, and MK-2. The ability to attach and proliferate was compared to control cells grown on new flasks.

Microwave Irradiation as a Method for Inactivating Viruses

Purified viruses (poliovirus TYPE I, OSU-T strain Sendai, T_4 bacteriophage) were diluted with BSS. A 1 ml aliquot of each virus suspension was placed into a 2 ml-vial (1 X 4 cm^2) and subjected to microwave irradiation for 10 to 180 seconds with 10 seconds rest intervals. After irradiation a plaque assay was performed for each virus suspension. After each exposure the microwave oven was cooled and the heat sink changed.

Comparison of Microwave Inactivated Sendai Vaccine with Commercial Sendai Vaccine

Commercial Sendai vaccine (formaldehyde inactivated Sendai, M. A. Bioproducts, Walkerville, Maryland) was thawed at room temperature just prior to use. The titer of commercial Sendai vaccine was 128 HA/ml. OSU-T strain Sendai stock was diluted to 128 HA/ml. A 1 ml volume of OSU-T Sendai virus was added to a 2 ml-vial and subjected to microwave irradiation for 140 seconds.

Inbred BALB/C strain mice were pretitered for antibodies against OSU-T strain Sendai. Each mouse was given a single 0.1 ml intra-peritoneal dose of either commercial or test vaccine. Challenges tests were performed 5 weeks post vaccination by injecting 0.1 ml (2.56 HA) of turkey source Sendai intra-peritoneally. Mice were sacrificed at 7 days post challenge, and sera were collected and titered by the HI, CF assays.

CHAPTER III

RESULTS

Plastic Tissue Cultureware Sterilization With Microwave for Reuse

Experiments using plastic tissue cultureware were performed to see if they could withstand the microwave process. All vessels used, could withstand times up to and in all cases exceeding 10 minutes. However it was necessary to include a small beaker of water (250-500 ml.) in the microwave oven as a heat sink. It was also necessary to let the glass plate of the microwave oven cool between runs in order to prevent melting of the vessels.

One problem was encountered with vessels containing black caps such as the Lux 25 cm^2 tissue culture flasks. The black caps retain heat and tend to melt the tops of the flasks at treatment times over 5 minutes. This problem was solved by autoclaving the caps separately and by covering the mouth of the flasks with plastic wrap during the microwaving process. The soft, colored caps such as the Corning orange caps and the Falcon blue caps (roller bottles) presented no porblem during the microwaving process.

Results of the vessel contamination experiments are shown in Table I. All vessels were rendered sterile by 3 minutes of microwave treatment, when microorganisms were introduced into washed plastic

TABLE I

MICROWAVE STERILIZATION OF TISSUE CULTUREWARE
CONTAMINATED WITH SELECTED ORGANISMS

Growth of organisms	Microwave irradiation (sec.)							
	0	15	30	60	120	180	240	300
Pseudomonas fluorescens	+	+	+	+	-	-	-	-
Escherichia coli	+	+	+	+	-	-	-	-
Klebsiella pneumoniae	+	+	+	+	-	-	-	-
Proteus vulgaris	+	+	+	+	+	-	-	-
Streptococcus faecium	+	+	+	+	+	-	-	-
Sarcina lutea	+	+	+	+	-	-	-	-
Bacillus alvei	+	+	+	+	+	-	-	-
Bacillus globigii	+	+	+	+	+	-	-	-
Corynebacterium equi	+	+	+	+	+	-	-	-

+ and - : presence or absence of growth respectively.

flasks. Also the primary chicken embryo, primary turkey embryo, Hep-2, Vero, BGMK, and MK-2 cells have all been successfully grown in the recycled tissue cultureware. No difference could be observed between the control cells grown in sterile new tissue culture flasks and those grown in the recycled microwave sterilized flasks upon microscopic examination.

Polioviruses Identification and Characteristic Studies

Polioviruses were plaque purified three times as described in the Materials and Methods.

The formation of plaques with polioviruses of TYPE I, II, III could be seen 48 hours postinfection. Plaques appeared as round, uncolored areas contrasting with the red color of the surrounding living cells, stained with neutral red. The diameter of the plaques varied from 1 to 3 mm on each plate and were recognized according to size (LP-large plaque, MP-medium plaque, and SP-small plaque). Upon prolonged incubation time, the plaques of three types increased in size until they became confluent. The number of plaques however remained constant after 48 hours incubation.

During plaque purification, even though the size of chosen plaque was constant, the resultant plaque size of the same type of poliovirus varied. Three different types of viruses were isolated from the original plate (Table II).

Plaque formation with TYPE II virus could be prevented by pretreatment of the virus with LIM BENYESH-MELNICK serum pools B and E, where as pretreatment with other serum pools would not prevent plaque formation. Plaque formation with TYPE III virus could be prevented by

TABLE II

IDENTIFICATION OF UNKNOWN POLIOVIRUSES BY LIM BENYESH-MELNICK SERUM POOLS OF ENTEROVIRUSES

	POOL B	POOL E	POOL D	POOL H	TYPE I
LP	-	-	+	+	+
MP	+	+	+	+	-
SP	+	+	-	-	+

+ and -; presence or absence of plaques respectively

pretreatment of the virus with LIM BENYESH-MELNICK serum pools D and H, whereas pretreatment with other serum pools would not prevent plaque formation. Plaque formation with TYPE I virus could be prevented by pretreatment of the virus with LIM BENYESH-MELNICK polio monoserum pool whereas pretreatment with any other serum pools would not prevent plaque formation. According to the experiments, three different types of polioviruses were identified (Table II).

The titer was obtained by counting the plaque-forming units. At early passage TYPE I virus was found to have a titer of $10^{9.23}$ PFU/ml. The titer of TYPE III was $10^{8.89}$ PFU/ml. TYPE II had a titer of $10^{9.51}$ PFU/ml. After several passages all titers of poliovirus stocks had decreased one hundred-fold (Table III).

Virus Stocks

BGMK cells lend themselves to the production of constant high titer virus stocks. The highest titers, ranging between 10^{6} to 10^{7} plaque-forming units per ml suspernatant, were obtained 24 hours post-infection with a 10^{6-7} PFU of viral inoculum. Virus fluid was harvested when cells exhibited maximun CPE.

Changing the amount of inoculum did not change the resulting titer. The difference was that the time for maximun CPE was prolonged 24 to 48 hours, when the inoculum was reduced to half amount.

Microwave Inactivation of Viruses

For testing the survival of microwave treated viruses, one ml of each purified poliovirus TYPE I (10^{7} PFU/ml), OSU-T Sendai virus (10^{6-7} PFU/ml), and bacteriophage T_4 (10^{22} PFU/ml) in BSS was added to a 2-ml

TABLE III

TYPE AND TITER OF ISOLATED POLIOVIRUSES

	Type	Titer (PFU. ml^{-1})		
		Original	Early passage stock	Stock
LP	II	$1 \times 10^{4.5-5.5}$	$1 \times 10^{9.51}$	$1 \times 10^{7.1}$
MP	I	$1 \times 10^{5.4-6.4}$	$1 \times 10^{9.23}$	$1 \times 10^{6.6}$
SP	III	$1 \times 10^{5.2-6.2}$	$1 \times 10^{8.89}$	$1 \times 10^{7.2}$

vial and subjected to microwave irradiation at constant time intervals. Microwave treated samples were taken 0.1 ml to make 10-fold serial dilutions, each dilution sample was inoculated to proper cell monolayers in duplicate to search for development of plaques and to calculate virus titers of each sample.

Results of survival (log 10 PFU ml^{-1}) Vs. single vial exposed at regular time intervals are shown in Figure 1. The inactivation curves of three virus types revealed the same decreasing pattern.

The HA titer of treated OSU-T Sendai virus and the antigens of poliovirus reacting with complement fixing antibody of treated poliovirus TYPE I were also tested (Figure 2 and Table V). Hemagglutination by Sendai virus was inhibited with microwave exposures over 70 seconds, the decreasing HA curve was consistent with plaquing experiments, however, hemagglutination units decreased faster than plaque forming units. In the case of polio, CF antigen titers did not appear much different than heat treated, 56° C for 30 minutes, antigens of poliovirus TYPE I (Table IV) reported by Bouvier et al. (6) and Sawicki et al. (39).

Sendai Vaccination Studies

In this investigation, comparison of commercial Sendai vaccine and microwave inactivated OSU-T Sendai vaccine upon antibody production was made. One group of mice injected with commercial Sendai vaccine, another group of mice injected with microwave inactivated vaccine. Mice were housed individually in plastic cages in our laboratory. All mice survived throughout the experiments.

During seven weeks observation, no disease symptoms developed. Preexposure sera were collected and titered. Final serum samples were

TABLE IV

COMPLEMENT FIXATION TEST USING HEAT- AND MICROWAVE TREATED POLIO TYPE I VIRUS

	Antigen Inactivation								
		Heat treated	Microwave irradiation (time in sec.)						
Dilution serial	Untreated	56° C, 30 minutes	20	40	60	80	100	120	130
1:2	+	+	+	+	+	+	+	+	+
1:4	+	+	+	+	+	+	+	+	+
1:8	+	+	+	+	+	+	+	+	+
1:16	++++	++++	++++	++++	++++	++++	++++	++++	++++
1:32	++++	++++	++++	++++	++++	++++	++++	++++	++++
1:64	++++	++++	++++	++++	++++	++++	++++	++++	++++
1:128	++++	++++	++++	++++	++++	++++	++++	++++	++++

\+ Partial lysis

++++ Complete lysis

Ag: Poli Type I infected tissue culture fluid (10^7 pfu. ml^{-1})

Ab: Standard amount, 1:10 dilution of 640 U/ml antipolio virus antibody

Figure 1. Inactivation curves for three representative viruses after microwave treatment. One ml volumes of the virus suspensions were treated in the microwave oven for each of the times represented. The treated samples were then plated onto their respective host cells as described in the Materials and Methods. Control samples are represented in the figure as the zero time treatment. All virus samples were inactivated within 180 sec. exposure.

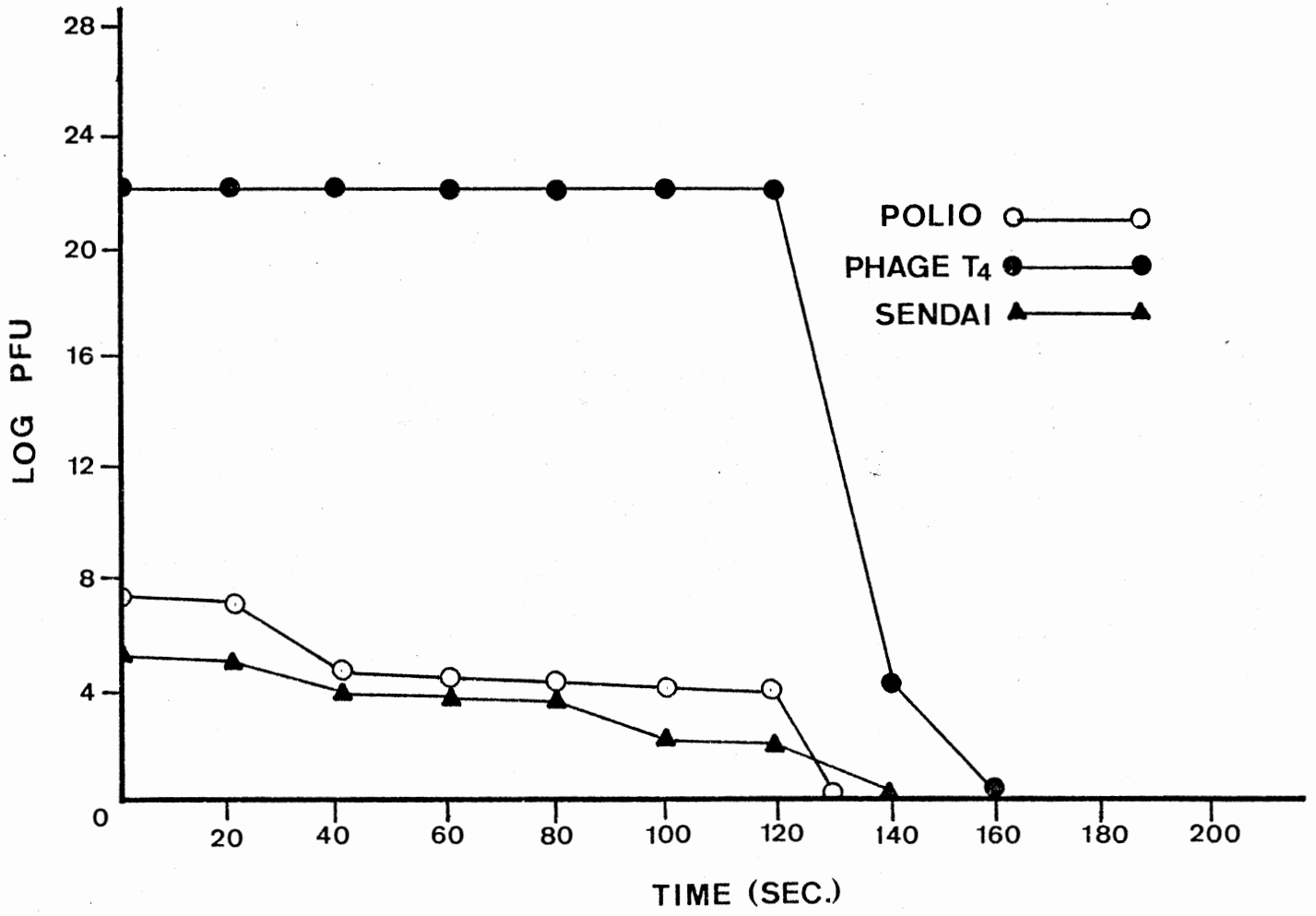
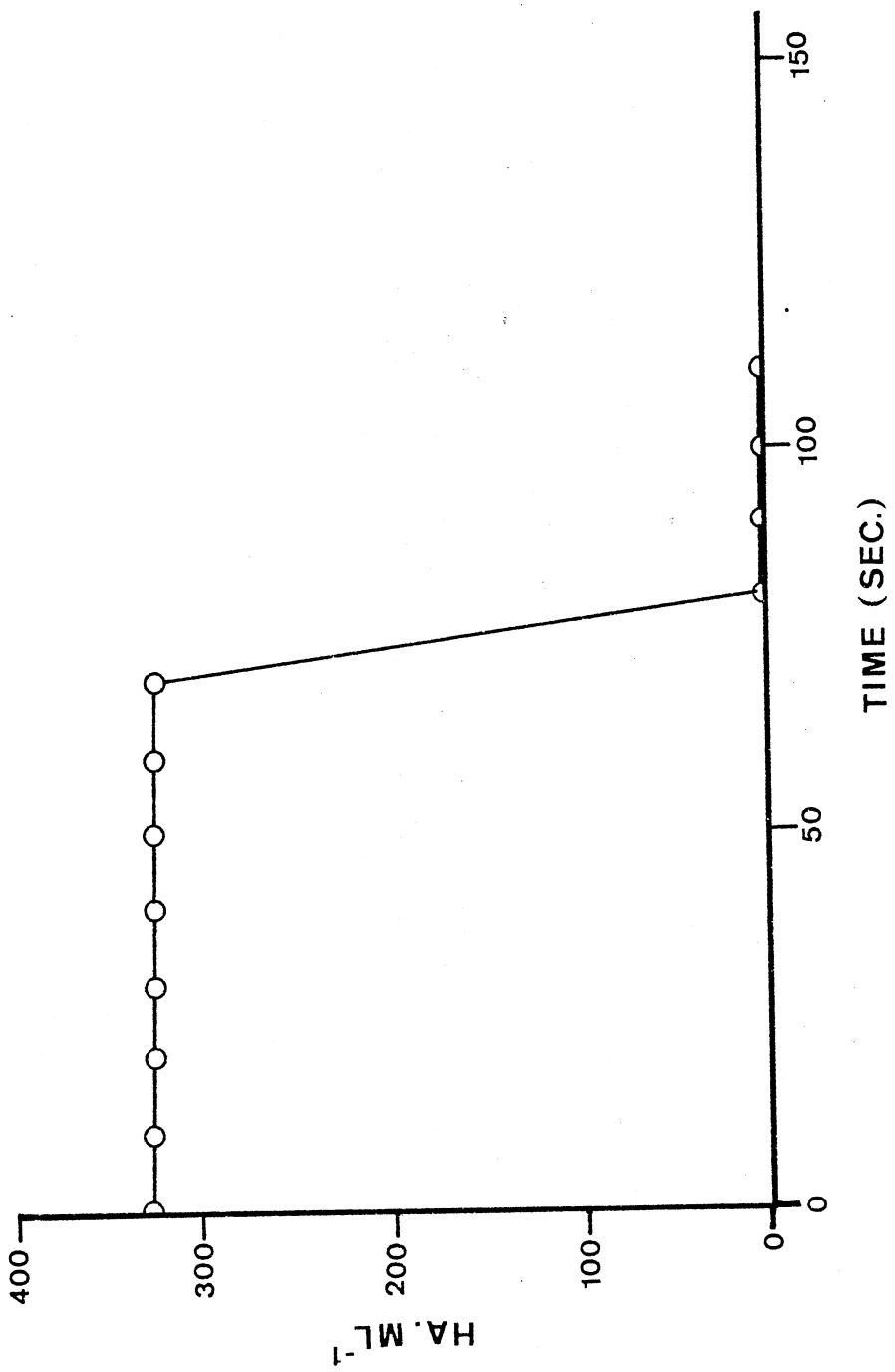

28
24
20
16
12
8
4
0
LOG PFU
20
40
60
80
100
120
140
160
180
200
TIME (SEC.)
POLIO
PHAGE T4
SENDAI

Figure 2. Microwave inactivation of OSU-T Sendai virus Hemagglutinin. Hemagglutination tests were run for each of the microwave treated OSU-T Sendai as described in the Materials and Methods. Control samples are represented in the figure as the zero time treatment.


HA. ML-1
400
300
200
100
0
0
50
100
150
TIME (SEC.)

collected seven days after vaccine challenge. No gross lesions on the mouse lungs were observed when autoptical examination was performed.

It has been noticed that both OSU-T Sendai virus and C-Sendai virus reacted with NIH standard anti-Sendai antibody by HI titration, which meant that the cross-reaction between different strains (different characteristics) of Sendai virus occurred (personal communication with M. R. Sanborn). However in our Sendai immunization studies, no antibody from mice immunized with OSU-T Sendai virus could be shown by HI tests (Table V). Antibodies to C-Sendai virus were found in two mouse serum samples. From the complement fixation test, no complement binding antibodies to C-Sendai virus were detected, but complement binding antibodies to OSU-T Sendai virus were shown in all serum samples except four control mice.

TABLE V

EXPERIMENTAL RESULTS OF SENDAI VACCINATION

No.	Strain	Sex/age (month)	Vaccine	Prevaccination HI titer	Postvaccination Ab titer To T-Sendai HI	To T-Sendai CF	To C-Sendai HI	To C-Sendai CF	Death Survival
1	BALB/C	♀ / 7	0.1 ml/ IP,CV	0	0	2	8	0	S
2	BALB/C	♀ / 7	0.1 ml/ IP,CV	0	0	2	0	0	S
3	BALB/C	♂ / 4	0.1 ml/ IP,CV	0	0	4	8	0	S
4	ICR(2)	♀ / 13	0.1 ml/ IP,CV	0	0	2	0	0	S
5	BALB/C	♀ / 4	0.1 ml/ IP,CV	0	0	4	0	0	S
6	BALB/C	♂ / 4	0.1 ml/ IP,TV	0	0	2	0	0	S
7	BALB/C	♀ / 4	0.1 ml/ IP,TV	0	0	4	0	0	S
8	BALB/C	♀ / 4	0.1 ml/ IP,TV	2	0	4	0	0	S
9	BALB/C	♀ / 4	0.1 ml/ IP,TV	2	0	2	0	0	S
10	BALB/C	♀ / 4	0.1 ml/ IP,TV	0	0	2	0	0	S
11	ICR(2)	♀ / 13	IP Challenge		0	0	0	0	S
12	ICR(2)	♀ / 13	IP Challenge		0	0	0	0	S
13	BALB/C	♀ / 7	IN Challenge		0	0	0	0	S
14	BALB/C	♀ / 7	IN Challenge		0	0	0	0	S

CV: Commercial vaccine
TV: Testing vaccine (microwave inactivated OSU T-Sendai)
T-Sendai: Turkey source Sendai
C-Sendai: Chicken source Sendai
IP: Intraperitonel injection
IN: Intranasal instillation

CHAPTER IV

DISCUSSION

Microwave Sterilization of Plastic Tissue Cultureware

Our experiments have shown that microwave irradiation is a practical method for sterilization of plastic tissue cultureware. This method is of particular value for laboratories which would benefit from the reuse of plastic cultureware. We have found that, for the vessels tested, all commercially available tissue cultureware is reuseable after microwave sterilization.

The killing times for the organisms tested generally agree with those reported by Latimer *et al.* (19), Goldblith *et al.* (17), and Culkin *et al.* (11). In the case of the *Bacillus* species the cultures did contain free spores and sporulating cells. It is interesting to note that the killing times for the organisms in our system was not much different from the other organisms tested. We have tested spore suspensions of the two *Bacillus* species and found them to be much more resistant to microwave irradiation, particularly when dry. Latimer's spore strips experiments showed that 1.3×10^9 *B. subtilus* spores per ml required exposure times of up to 11 minutes and *B. stearothermophilus* strips required exposure time within 5 minutes for a complete kill (19).

When considering the reuse of tissue cultureware it should be noted that the normal level of contamination, after washing, is much lower than that of our test contaminated vessels. Therefore the sterilization times should be adequate for normal reuse. Low levels of contamination by spores normally would not present a problem if they escaped inactivation since most tissue culture media contain antibotics to prevent organisms from growing. Flasks have been recycled more than five times with no significant effect on cell attachment or poliovirus plaque formation.

The non-sterile flasks, washed for reuse, were naturally contaminated with fungi. It was noted that fungal contamination was also eliminated with 180 seconds of microwave irradiation. No attempt was made to identify these contaminates and no anti-fungal agents were included in the culture medium.

Recently concern has been raised about cross contamination of tissue culture lines with other cell lines (25). This concept becomes a real concern when one contemplates the reuse of tissue cultureware. We felt that even if residual cells escape the washing process, the microwave sterilization process would kill them. In support of this contension Patterson and Bulard (32, 33) have shown tissue culture cells to be adequately fixed for fluorescent antibody staining after only 12 seconds of microwave irradiation.

If flasks were contaminated with viruses, could they be washed, disinfected, and sterilized by microwave for reuse? Concerning this problem, we completed the studies of microwave inactivation of viruses. Fgiure 1 shows that three types of viruses were indeed inactivated by microwave exposure within 140 seconds. The flasks for viral propagation

were treated with "Clorox" disinfectant, and then followed the washing and sterilization procedures. Apparently, the washing and microwave processes do not damage the attachment properties of the plastic to any great extent; also no virus contamination occurred.

From our experience we have generated the following general protocol for the reuse of plastic tissue cultureware. First the vessels are washed with a detergent followed by adequate washes with tap water, then rinse with deionized water four times. The vessles are allowed to dry overnight. Caps, when using flasks, are loosened about one turn prior to sterilization. If the caps are of the black type they are autoclaved and added to the flasks after microwave treatment. Tissue culture is then placed in the microwave oven along with a flask of water (250-500 ml) which serves as a heat sink. During one run no more than 12 flasks are placed in the oven. We have selected a 10 minutes treatment time for the vessels. This insures that all areas within the oven receive enough energy. The home type microwave ovens are noted for their uneven heating depending upon where the load is placed in the oven. If one is sterilizing several loads it is best to let the inside of the oven cool before adding the subsequent loads in order to prevent the plastic from reaching too high a temperature.

We have found that microwave sterilization is a rapid and economical way to sterilize plastic tissue cultureware for reuse. By recycling three to four cases of 75 cm^2 flasks one can pay for a more than adequate microwave oven. This feature will become more prominent as the price of plastic ware increases.

Characteristics of Polioviruses

Virus stocks of polio have been propagated or detected on primary cell cultures of African green or rhesus monkey kidneys, and on HeLa, Vero, Hep-2 or BGMK cell lines (2, 12, 14, 24). We found that during early experiments HeLa, Vero, and Hep-2 cell lines, passaged routinely in our laboratory were inadequate for viral multiplication. We were unable to show plaque formation and cytopathic effects on infected cells. In additional tests it was found that BGMK cells (courtesy of Dr. G. Wallis, Baylor Medical Center, Texas) provided best results for our poliovirus studies because of their uniform susceptibility for viral infection and plaque production (12).

In order to determine whether the difference in plaque sizes that was observed was genetic, stocks from large, medium and small plaques were plated separately and scored for plaque size. The plaques produced by stocks of all types were indistinguishable and showed again the same variability in plaque size. Dulbecco et al. (14) have published similar findings. It was, therefore, concluded that differences in plaque size were non-hereditary even though at the beginning of our studies isolates of polioviruses were size-dependent. Dulbecco et al. also proposed that plaque size may be attributed to differences in time of adsorption of individual particles onto the cells of the same culture or part of unknown causes.

Oral live polioviruses vaccine contains three types of polioviruses. No cross-reaction occurred between different types of pure polioviruses and their serum pools (35). Plaque pure virus stocks were established by isolating the virus population produced in single

plaques. The virus stocks have been serotyped with LIM BENYESH-MELNICK serum pools, and one plaque purified stock was isolated for each type.

From our experimental procedure the titer of polioviruses were not increased by heavy doses infection of any type of polioviruses. The titer in plaque-forming units of TYPE I was 1 X $10^{6.6}$, TYPE II was 1 X $10^{7.1}$, and TYPE III was 1 X $10^{7.2}$ (Table III). The constant titers were obtained after all when purest virus had been isolated from the specimen. The question remains as to why the PFU titers of early propagated polioviruses were higher than the later propagated polioviruses. The titers of virus stocks increased only 10-fold after proper concentrating procedures. One explanation might be that poliovirus tend to clump when concentrated, particularly in the presence of Ca^{++} and Mg^{++}. Thus a plaque-forming unit might actually contain more than one physical virus particle.

The final observation from this study was that plaque assay provided a very sensitive method for detecting polioviruses. To compare the differences in titers of both PFU method and CPE method, the titer obtained by CPE was usually 10-fold lower than PFU. The possible reason for this may be that when highly diluted virus was inoculated on BGMK cell monolayers for CPE assay, all-or-none CPE reaction would not occur during the period of observation, which would confuse us on the reading of the end point titer.

Comparison of the Efficiency of Microwave Inactivated Sendai Virus Vaccine and Commercial Sendai Virus Vaccine

Sendai virus is one of the more prevalent and serious virus infections in mouse, hamster, rat, and guinea pig colonies throughout the world (36, 37, 41). Disease and death have been well studied only for mice. In the United States, Sendai virus infection of mice was first found to be wide spread by Parker et al. (30). According to the serological studies on randomly selected mouse serum samples, it was found that 44% colonies of mice normally contained detectable anti-Sendai antibody which indicated that those mice were found to be frequent contaminants (29).

Because of the widespread occurrence of the virus and its potential silent infection of the mouse respiratory system, mice generally used in the laboratory should be immunized with Sendai vaccine to insure that experimental results are not affected by this undesirable agent.

Since formaldehyde inactivated Sendai vaccine is the only licensed vaccine on the market, we were interested in developing a microwave inactivated Sendai vaccine for completing a part of our microwave studies. In order to determine whether the antigenicity of microwave inactivated OSU-T Sendai virus, in vivo, still remained or was totally destroyed by microwave energy, ten non-immune mice were separated to two groups and injected either with microwave inactivated Sendai virus or with commercial Sendai vaccine. At the end of experiment antibody titers were detected by HI and CF titration. Table V revealed that HI titers were

very low or none for all control and test mice. It seemed that injected Sendai virus vaccine did not stimulate mouse immune systems to produce antihemagglutinin antibody. However it was found that complement-fixing Abs were produced in both groups. From CF titers it was shown that there was no distinguishable difference between control vaccine and test vaccine. According to this preliminary study we concluded that microwave inactivated Sendai vaccine was comparable to the commercial Sendai virus vaccine.

The envelope of Sendai virus, which is a modified host-cell membrane, contains projections of viral glycoproteins which have hemagglutinating and neuraminidase activities for hemagglutination and viral absorption. In our study we found that the hemagglutinating ability of microwave inactivated OSU-T Sendai virus was inhibited after 80 seconds microwave exposure, and it required 140 seconds exposure for plaque inhibition.

According to Parker's (31) data, inbred strains of mice showed wide variation in their sensitivity to infection with Sendai virus. The data revealed that the infectivity titers in mice of all tested strains were nearly identical regardless of their sensitivity to lethal infection. If one considers lethal infections, the difference in the dosage required between some strains was 25,000-fold. Their data indicated that the lethal infection in BALB/c strain was induced with 100 $TCID_{50}$. In our experiment both immune mice and challenge control mice were inbread BALB/c strain, all mice were challenged by intraperitoneal injection or intranasal installation 2.56 HA or 12.8 HA Sendai virus per mouse respectively. This activity represents 2.56×10^5 PFU and 1.28×10^6 PFU per mouse respectively, which was 100 to 1,000 times higher than the

dosage that Parker used to inject their BALB/c strain. However our experimental mice did not die after challenge and also did not show any symptoms of disease. The one possible explanation was that age of experimental mice was too high in our case. Because Sawicki (39) has proved that the susceptibility of Sendai virus infection on mouse was age-dependent. Younger mice were more susceptible to lethal infection than subadults or adults.

Summary

Microwave energy at 2,450 MHz is able to kill bacteria and to inactivate viruses within 160 seconds exposure. These results indicated that microwave irradiation provided a good method to sterilize plastic tissue cultureware for reuse. Varieties of plastic tissue cultureware were solely subjected to microwave irradiation treatment for 10 minutes without changing cell attachment properties.

Primarily experimental data revealed that the antigenicity of microwave treated Sendai virus *in vivo* was not altered in the yield of complement-fixing AB compared to the commercial formaldehyde inactivated Sendai virus vaccine.

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